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**Comparative metagenomic analysis of electrogenic microbial communities in differentially inoculated swine wastewater-fed microbial fuel cells**

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**Key words:** metagenomic analysis; microbial fuel cell; agricultural wastes; wastewater treatment; volatile fatty acid removal; bioreactors; microbial inoculum; pig slurry.

## 27 Abstract

28 Bio-electrochemical systems such as microbial fuel cells (MFCs) are promising new  
29 technologies for efficient removal of organic compounds from industrial wastewaters, including that  
30 generated from swine farming. We inoculated two pairs of laboratory-scale MFCs with sludge  
31 granules from a beer wastewater treating anaerobic digester (IGBS) or from sludge taken from the  
32 bottom of a tank receiving swine wastewater (SS). The SS-inoculated MFC outperformed the IGBS-  
33 inoculated MFC with regard to COD and VFA removal and electricity production. Using a  
34 metagenomic approach here we describe the microbial diversity of the MFCs planktonic and anodic  
35 communities derived from the different inocula. *Proteobacteria* (mostly *Deltaproteobacteria*) became  
36 the predominant phylum in both MFCs anodic communities with amplification the electrogenic genus  
37 *Geobacter* being the most pronounced. Eight dominant and three minor species of *Geobacter* were  
38 found in both anodic communities of the MFCs. The anodic communities of the SS-inoculated MFCs  
39 had a higher proportion of *Clostridium* and *Bacterioides* relative to those of the IGBS-inoculated  
40 MFCs, which were enriched with *Pelobacter*. The archaeal populations of the SS- and IGBS-  
41 inoculated MFCs were dominated by *Methanosarcina barkeri* and *Methanothermobacter*  
42 *thermautotrophicus*, respectively. Our results thus show a long-term influence of inoculum type on the  
43 performance and microbial community composition of swine wastewater-treating MFCs.

44

## 45 Introduction

46 Livestock farming constitutes an important agricultural sector of many countries but produces  
47 considerable amounts of organic wastes that require proper treatment and disposal. The rapidly  
48 growing pig farming industry generates high-strength wastewater containing organic compounds,  
49 ammonia, phosphates, odorous gases, suspended solids, and pathogens [1]. Treating swine wastewater  
50 is especially difficult where land is limited pig farming facilities occur in close proximity to population  
51 centers, such as in Okinawa, Japan. The lack of available land for application of swine wastewater

(SW) as a fertilizer and potential for contamination of surface and ground water sources underscores the need to employ thorough treatment of SW.

Common methods of treating SW include aerobic oxidation ponds, lagoons, anaerobic digestion and constructed wetlands [2]. Bio-electrochemical systems such as microbial fuel cells (MFCs) are promising new technologies for efficient removal of organic compounds in wastewaters. Inside the confined anaerobic chamber of an MFC a consortium of bacteria catalyze oxidation reactions, depositing electrons on the anode by a variety of means, including directly via outer membrane proteins or conductive pili or indirectly via secretion and recycling of redox-active molecules [3].

A primary target of SW treatment is a set of volatile fatty acids (VFAs) largely responsible for its noxious odor [4]. The presence of VFAs in an MFC substrate can increase the electrogenic performance of its anodic microbial biofilm [5]. Laboratory-scale single batch-loaded MFCs have been shown to dramatically lower malodorous compounds (primarily VFAs) as well as other constituents present in SW [4].

One important determinant of MFC reactor performance is the composition of the microbial community in the anodic chamber [6]. For obtaining maximal initial power production the anodic biofilm of an existing MFC has been shown to serve as a better inoculum than anaerobic sludge but we know of no study that assesses inoculum performance relative to pollutant removal criteria [7]. To this end, we sought to determine whether a microbial community already familiar with a SW substrate would perform better in an MFC than a distinct beer waste-digesting anaerobic sludge, assessing treatment performance and microbial community composition.

Previous studies have assessed microbial community composition in SW-fed MFCs utilizing denaturing gel gradient electrophoresis while a more recent study has utilized high-throughput amplicon sequencing to examine influences of external resistance and hydrodynamics on the MFC microbiome [8, 9]. Using a metagenomic approach here we describe the microbial diversity of the MFCs planktonic and anodic communities derived from the different inocula. Clustering of microbial

77 communities based on dominant bacterial genera indicates that the nature of the inoculum is an  
78 important influence on the ultimate composition of microbial communities and performance of MFCs.

79

## 80 **Materials and Methods**

### 81 **MFC configuration and operation**

82 The internal MFC chamber contained two anodes (approximately 6 x 8 cm), suspended 2-3 mm  
83 off the bottom of the chamber, composed of a layer of conductive carbon cloth to which 2 mm average  
84 size activated carbon granules were bound with conductive glue to provide more surface area. The  
85 granules had been prepared from birch precursor and were pre-treated with a neutral red catalyst. The  
86 two cathodes were graphite plates (3 mm thick; 60% porosity) sprayed on the liquid-facing side with  
87 an aqueous 5% Fumion membrane polymer (Fumatech, Bietigheim-Bissingen, Germany) while  
88 activated carbon granules [treated with iron(II) phthalocyanine] were mechanically pressed to the air-  
89 facing side using netting frame. The cathode extended into a bath containing an electrolyte solution  
90 (maintained at pH 3 with regular additions of 0.1N HCl).

91 The anode and cathode electrodes were connected with a multi-channel logger (Graphtech midi  
92 LOGGER GL820, Japan) for daily voltage measurements. The corresponding electric current was  
93 calculated using Ohm's law ( $V=IR$ ). Power density was obtained according to  
94  $P = \frac{IV}{A}$ , where I is the current, V is the voltage, and A is the projected surface area of the cathode.  
95 Polarization and power curves obtained by changing external resistances (from 0  $\Omega$  to 2100  $\Omega$ ) in open  
96 circuit when the values had stabilized at each resistance indicated an internal resistance of 70  $\Omega$  within  
97 the MFCs (Fig S1).

98 For inoculation of the MFCs swine wastewater sludge (SS) containing suspended scrapings from  
99 the bottom of the SW holding tank was collected from a local pig farm (Okinawa Livestock and  
100 Grassland Centre, Nago, Japan) and industrial granular brewery sludge (IGBS) from a wastewater-  
101 treating UASB reactor (Orion Brewery, Nago, Japan). The inocula were not chemically modified or

102 diluted though the SS inoculum was filtered through a 1 mm stainless steel mesh.

103 To allow for microbial biofilm formation two MFCs were inoculated with SS and two with IGBS,  
104 heretofore referred to as SS-MFC and IGBS-MFC, and allowed to sit for 3 days in open-circuit mode  
105 at room temperature (24°C). The MFCs were then provided undiluted SW in fed-batch mode to  
106 achieve a 24 h HRT. Regular feeding and monitoring of electrical performance began 13 d following  
107 inoculation of the MFCs, coincident with the switch to closed-circuit mode.

108 SW for use as MFC feed was stored at 4°C. To remove large particles, the raw SW was sieved  
109 through a 0.50 mm mesh (Nylon monofilament). SW feed was diluted with distilled water to adjust the  
110 chemical oxygen demand (COD) to 3.5–7.4 g O<sub>2</sub> L<sup>-1</sup> and the hydraulic retention time (HRT) set to 1 or  
111 2 d over the course of the experiment. Wastewater was added into the MFCs semi-continuously using a  
112 peristaltic pump (Masterflex L/S Precision pump, Cole-Parmer, USA) set to a 6 ml min<sup>-1</sup> flow rate.  
113 Operational parameters for the MFCs over the course of the 67-day experiment are summarized in  
114 Table S1.

## 115 **Chemical analyses**

116 Sampling of MFC inflow and outflow was performed every 24 h. COD, volatile fatty acids  
117 (VFA), ammonia nitrogen (NH<sub>3</sub>-N), and total phosphorus (PO<sub>4</sub><sup>3-</sup>-P) determinations were measured  
118 using the HACH TNTplus Chemistries (HACH Company, Loveland, CO). Total COD of inlet swine  
119 wastewater and MFC-treated effluent was measured without filtration. pH was measured with a pH  
120 meter (Horiba D-51, Japan).

## 121 **Chromatography**

122 Specific VFA compounds were quantified using an Agilent 7890A gas chromatograph  
123 connected to a LECO Pegasus 4D TOF mass spectrometer. Separation of VFA was performed using a  
124 Stabilwax-DA (30 m, 0.25 mm ID, 0.25 µm) column, using helium as carrier gas at 1.11 ml min<sup>-1</sup> flow  
125 for the entire run. Method development was performed using Supelco WSFA-2 Mix to obtain retention  
126 index (RI) calibration and quantification calibration curve. Approximately 1 ml of sample was

transferred through a 0.22  $\mu\text{m}$  filter to a glass autosampler vial. A 1:20 split liquid injection (1  $\mu\text{l}$  volume) was injected, with the injection port set at 250°C, 1 ml min<sup>-1</sup> septum purge flow. The gradient temperature protocol was 2 min at 100°C followed by an increase to 145°C at a rate of 20°C min<sup>-1</sup>, holding at 145°C for 6 min, followed by an increase to 205°C at 20 °C min<sup>-1</sup> and holding this temperature for 4 min. The mass spectrometer was set with 35 to 145 Da mass scan range, 5 spectra sec<sup>-1</sup> acquisition rate, and -70V electron energy. Ion source and transfer line temperature was 250°C. Data processing (deconvolution, identification and quantification) was done using LECO ChromaTOF version 4.50.8 software. Acetic acid (99.99% purity), butyric acid (99.5%), 2-ethylbutyric acid (99%), hexanoic acid (99.5%), isovaleric acid (99%), isobutyric acid (99.5%), octanoic acid (99.5%), propionic acid (99.8%), sulfuric acid (99.9%), and valeric acid (99.8%) standards were purchased from Sigma-Aldrich, Japan.

## Microbial diversity analysis

DNA was isolated from swine wastewater, inoculum sludges, anodic biofilms (carbon felt and carbon granules) and planktonic samples of each MFC using PowerMax soil DNA isolation kit (MO BIO laboratories, Inc). DNA quality was evaluated by the Agilent 2100 Bioanalyzer system. A DNA library was constructed for shotgun sequencing and a 150 paired-end sequencing reaction was performed on MiSeq platform (Illumina, San-Diego, CA, USA).

The sequencing data were uploaded to the MG-RAST server as FASTAQ files for processing, primary analysis and storage. *Sus scrofa* (pig) genome sequences were marked for exclusion during data submission. Primary submission data and results of the MG-RAST pipeline are available publicly (project mgp19536). The MG-RAST representative hit organism abundances calculation was performed against the SEED database at the level of genera, based on a maximum e-value of  $1 \times 10^{-5}$ , minimum identity cut-off of 60% and minimum sequence alignment of 15. Abundance data were downloaded as TSV files for further analysis. The representative hit data were downloaded from MG-RAST server via MGRASTer package [<https://github.com/braithwaite/MGRASTer/>] in R 3.1

environment. Abundance analysis was performed in metagenome Seq package [10] and ordination analysis was performed with phyloseq R packages [11]. Krona taxonomic community profiles were built by MG-RAST and stored as an image.

155

## 156 Results

157 We applied an integrated approach to investigating the effect of two distinct inoculums on  
158 performance of MFCs treated SW, comparing source- and site-dependent differences in the diversity  
159 of the microbial community, electricity production, and removal of organics.

### 160 MFC performance characteristics

161 The SS-MFC pairs outperformed the IGBS-MFCs pairs in regard to electricity generation (Fig  
162 S2) and removal of COD and VFA from the SW feed (Table 1; Fig S3) while operating on a 48 h  
163 HRT. Both MFC pairs displayed negligible removal phosphate (Table 1, Fig S4) whereas the SS-MFC  
164 performed better than the IGBS-MFC at removing ammonia (Table 1, Fig S5). Over the 67 d course of  
165 the experiment the SS-MFC COD removal rate of  $2.65 \pm 0.11 \text{ mg O}_2 \text{ L}^{-1} \text{ d}^{-1}$  was slightly but  
166 significantly higher than the IGBS-MFC rate of  $2.26 \pm 0.17 \text{ mg O}_2 \text{ L}^{-1} \text{ d}^{-1}$  ( $p = 0.02$ ) while their  
167 respective VFA removal rates of  $0.76 \pm 0.06 \text{ mg L}^{-1} \text{ d}^{-1}$  and  $0.66 \pm 0.05 \text{ mg L}^{-1} \text{ d}^{-1}$  did not differ  
168 significantly ( $p = 0.27$ ; means  $\pm$  SE,  $n = 4$ ). Electrical output of the MFC pairs remained relatively  
169 stable over the course of the experiment with the current density of the SS-MFCs ( $56.6 \pm 2.4 \text{ mA m}^{-2}$ )  
170 being consistently higher than that of the IGBS-MFCs ( $43.5 \pm 6.2 \text{ mA m}^{-2}$ ) (means  $\pm$  SD,  $n = 43$ ; Fig  
171 S1).

172 **TABLE 1. Treatment-related characteristics of swine wastewater-fed microbial fuel cells.**

Source	COD (mg L <sup>-1</sup> ) <sup>[a,b]</sup>	VFA (mg L <sup>-1</sup> ) <sup>[a,b]</sup>	NH <sub>4</sub> <sup>+</sup> -N (mg L <sup>-1</sup> ) <sup>[a,b]</sup>	PO <sub>4</sub> <sup>3-</sup> -P (mg L <sup>-1</sup> ) <sup>[a,b]</sup>
SW inflow	6824	1452	365	374
SS-inoculated MFC	1684 (-75.3%)	222 (-84.8%)	286 (-21.6%)	365 (-2.4%)
IGBS-inoculated MFC	2219 (-67.5%)	314 (-78.4%)	327 (-10.4%)	370 (-1.1%)



173  
174 <sup>[a]</sup>Results are means of measurements taken of two independently operating MFC for both MFC  
175 types, both operating with 100  $\Omega$  external resistance sampled at 67 days following initiation of  
176 operations.  
177 <sup>[b]</sup>Percent change in parentheses.

178 Changes in the concentrations of straight-chain (acetic, propionic, butyric, valeric, hexanoic) and  
179 branched chain (isobutyric, isovaleric) VFAs were monitored (Table 2). Of these, propionic acid was  
180 found at the highest concentration in the SW. Passage through the SS-MFC removed >90% of all  
181 monitored VFAs, except for propionic acid, which was dissipated by 85.9%, and outperformed the  
182 removal rate of IGBS-MFC for all VFA tested. Predominance of propionic or acetic acids among  
183 VFAs in MFC-treated SW effluent has been previously shown [4, 12]. Several aromatic ring  
184 compounds (phenols and indoles) can contribute to the odor of SW [4], however we detected only two  
185 aromatic compounds (p-cresol and phenol) at negligible concentrations and indoles were not found  
186 (results not shown).

187 **TABLE 2. Removal of selected volatile fatty acids by swine wastewater-fed microbial fuel cells.**

	Concentration (mg L <sup>-1</sup> )*						
Source	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Hexanoate
SW inflow	114.95	425.26	10.40	198.07	81.48	127.26	96.56
SS- inoculated MFC effluent	7.06 ±3.65	59.98 ±51.37	0.90 ±0.78	0.80 ±0.46	5.62 ±4.79	0.98 ±0.88	0.51 ±0.29
IGBS- inoculated MFC effluent	8.78 ±3.72	108.65 ±25.54	6.24 ±1.60	4.50 ±1.12	40.89 ±7.41	12.26 ±3.90	4.87 ±0.83

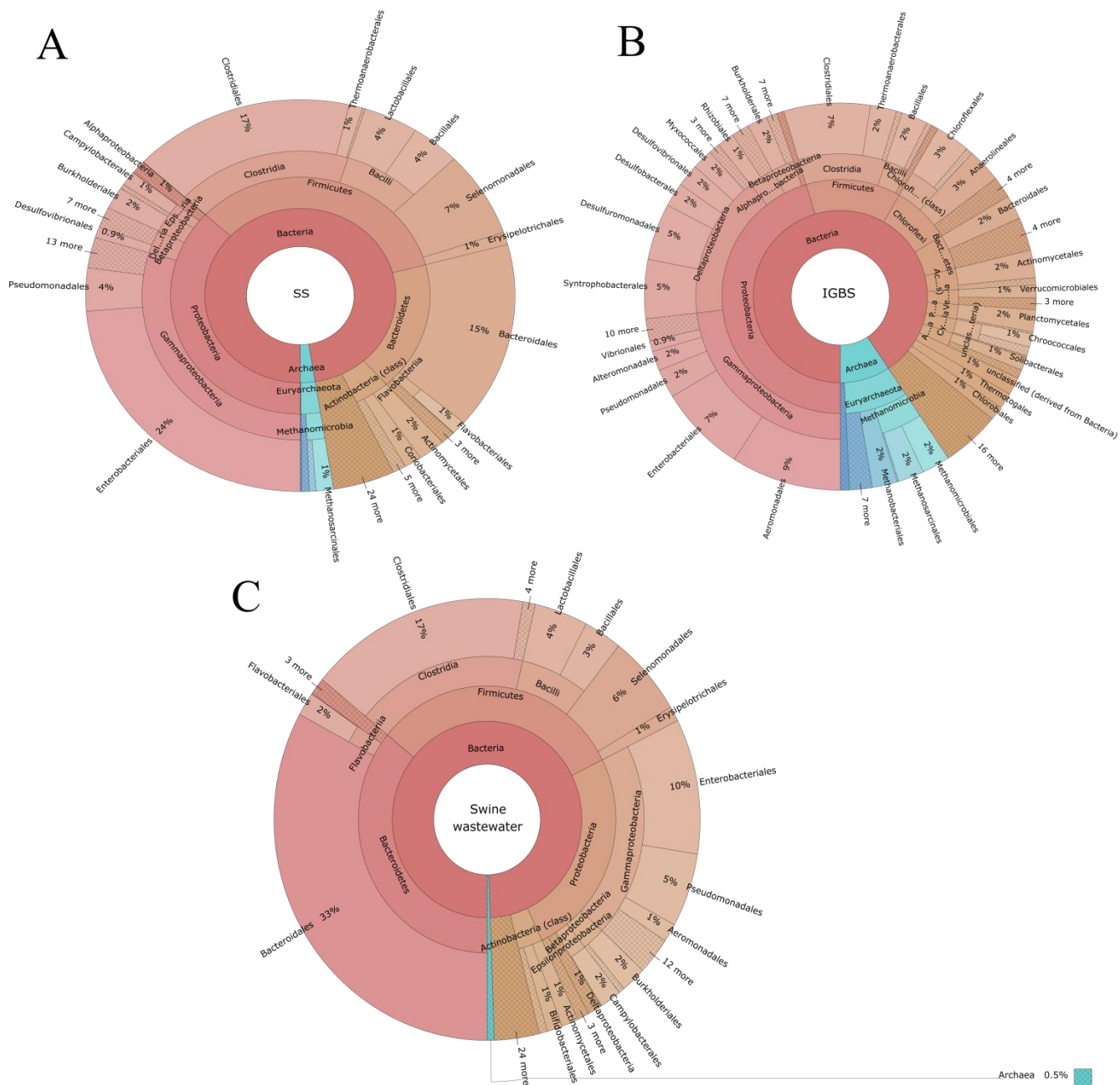
188 \* ± Range of variation between the two MFCs of each type.

## 189 Metagenomic analysis of microbial communities

### 190 Inocula

191 Over 98% and 91% of genes were affiliated with the domain *Bacteria*, and only 2% and 9% of  
192 genes were represented by *Archaea* for the SS and IGBS inoculums, respectively (Fig 1A-B). 30%  
193 *Gammaproteobacteria* (dominant genus *Enterobacteriaceae* (24%)) were the most abundant in SS

194 inoculum, whereas 23% *Gammaproteobacteria*, dominated by the genera *Aeromonadaceae* (9%) and  
 195 *Enterobacteriaceae* (7%), were identified in IGBS inoculum (Fig 1A-B). *Deltaproteobacteria* were  
 196 represented by the dominant genera *Desulfovibrionaceae* (0.8%) in SS inoculum and *Geobacteraceae*  
 197 (3%) and *Syntrophaceae* (3%) in IGBS inoculum (Fig 1A-B).



198

199 **Figure 1. Summary of the microbial community profiles in the multilevel Kona diagrams.**

200 Kona plots visualizing taxonomic hierarchies of the microbial communities of (A) swine sludge (SS),

201 (B) industrial granular brewery sludge (IGBS), and (C) swine wastewater.

202 The phylum *Firmicutes* (34% (SS) and 13% (IGBS)) was represented by *Clostridia* and *Bacilli*  
203 classes in both inoculums (Fig 1A-B). The most abundant members of phylum *Bacteroidetes* (18%)  
204 were identified as *Prevotellaceae* (8%) and *Bacteroidaceae* (5%) in SS inoculum (Fig 1A). Despite the  
205 low content of *Bacteroidetes* in IGBS inoculum (5%) the diversity of bacterial families was similar to  
206 that of the SS inoculum (Fig 1B). On the other hand, IGBS inoculum was enriched by *Chloroflexi*  
207 (8%), with dominant members *Anaerolineaceae* (3%) and *Chloroflexaceae* (3%) (Fig 1A-B).  
208 *Actinobacteria* (*Actinomycetales*) was found to be relatively abundant in both inoculums (Fig 1A-B).  
209 *Cyanobacteria* (*Chroococcales*) with abundance >0.5% were detected in SS inoculum, while 2% were  
210 identified in IGBS inoculum (Fig 1A-B).

211 Phylum *Archaea* was more abundant in the IGBS inoculum (9%) compared to the SS inoculum  
212 (2%) (Fig 1A-B). Analysis of two inoculums showed that *Euryarchaeota* (*Methanosarcinales* (1%)  
213 and *Crenarchaeota* (*Desulfurococcales*) were represented in both inoculums (Fig 1A-B). Thus, two  
214 types of inoculums were detailed analyzed aimed to investigation formation of the electrogenic  
215 microbial communities of MFCs with a highly effective treatment and degradative ability.

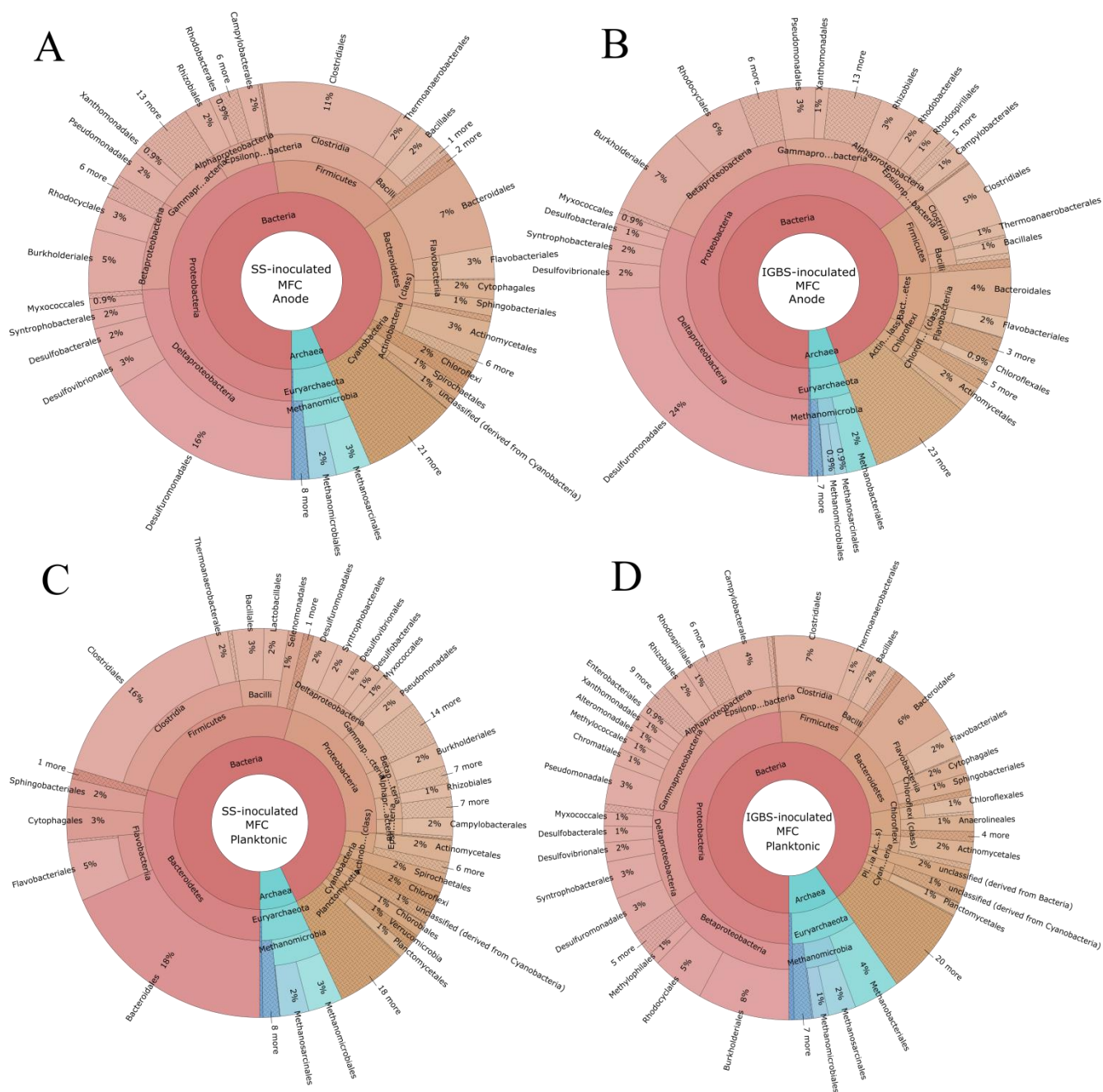
## 216 **Swine wastewater**

217 The microbial community analysis of SW showed that *Bacteroidetes* (36%), *Firmicutes* (32%),  
218 *Proteobacteria* (25%), *Actinobacteria* (3%) and 24 classes of *Bacteria* with relative abundance >1%  
219 were present (Fig 1C). The *Proteobacteria* were composed of *Gammaproteobacteria* (19%)  
220 (predominantly *Enterobacteriaceae* (10%)), *Epsilonproteobacteria* (2%) (with *Campylobacteraceae*  
221 (1%)), *Deltaproteobacteria* (1%) (with *Desulfovibrionaceae* (0.4%)) and *Alphaproteobacteria* (0.9%)  
222 (with *Rhizobiales* (0.4%)) (Fig 1C). Phylum *Firmicutes* and *Bacteroidetes* of the SW microbial  
223 community had a distribution of dominant members similar to the SS inoculum (Figs 1A-C). Archaeal  
224 communities representing 0.5% of the total detected bacteria were dominated by classes of  
225 methanogens *Methanomicrobia* and *Methanobacteria* (Fig 1C).

226

227 **MFC anodic microbial communities**

228       The microbial diversity of anodic communities was similar between the SS-MFCs and IGBS-  
229 MFCs at the level of genera (Fig 2). *Deltaproteobacteria* reached up 24% and 31% of the total  
230 microbial population on the anodes of SS-MFCs and IGBS-MFCs, respectively (Fig 2C-D). Among  
231 the *Deltaproteobacteria* class, *Geobacter* (14% and 20%) was identified as the most highly abundant  
232 genus on both MFC anodes.



**Figure 2. Summary of the anodic and planktonic microbial community profiles in**

**multilevel Krona diagrams.** Krona plots visualizing taxonomic hierarchies of the microbial communities of (A) swine sludge-inoculated MFC anode, (B) industrial granular brewery sludge-inoculated MFC anode, and (C) swine sludge-inoculated MFC planktonic contents, and (D) industrial granular brewery sludge-inoculated MFC planktonic contents.

The *Gammaproteobacteria*, common in both inocula (7% of SS and 8% of IGBS) and SW (30%), were substantially less represented on the anodes of both MFCs (Fig 2C-D). Particular declines in the *Enterobacteriaceae* ~~led~~—corresponded with higher relative levels of *Moraxellaceae* (*Acinetobacter*), *Pseudomonadaceae* (*Pseudomonas*) and *Xanthomonadaceae* (*Xanthomonas*) among the *Gammaproteobacteria* in the anodic community of SS-MFCs; and *Pseudomonadaceae* (*Pseudomonas*) and *Xanthomonadaceae* (*Xanthomonas*) in the anodic community of IGBS-MFCs (Fig 2C-D). Known electrogenic bacteria *Shewanella* (*Shewanellaceae*) were found on the anodes of both MFCs (Fig 2C-D).

Phylum *Firmicutes* (*Clostridia* and *Bacillus*) occupied only 13% and 9% of the total microbial population in SS-MFC and IGBS-MFC anodic communities, less compared to the inocula and SW (Fig 2C-D). Slight increases in the proportion of *Bacteroidetes* members (*Bacteroidales* and *Flavobacteriales*) (19%) were observed in the SS-MFC anodic communities compare to the inoculum (Fig 2C-D). Members of phylum *Chloroflexi* (*Roseiflexus* and *Anaerolinea*) were enriched in the population of anodic microbial community of SS-MFC, whereas that of the IGBS-MFC had less *Chloroflexi* (Fig 2). Slight enrichment of facultative heterotrophic *Cyanobacteria* genera (*Cyanothece*, *Synechococcus* and *Nostoc*) on anodes of both MFCs was detected (Fig 2C-D).

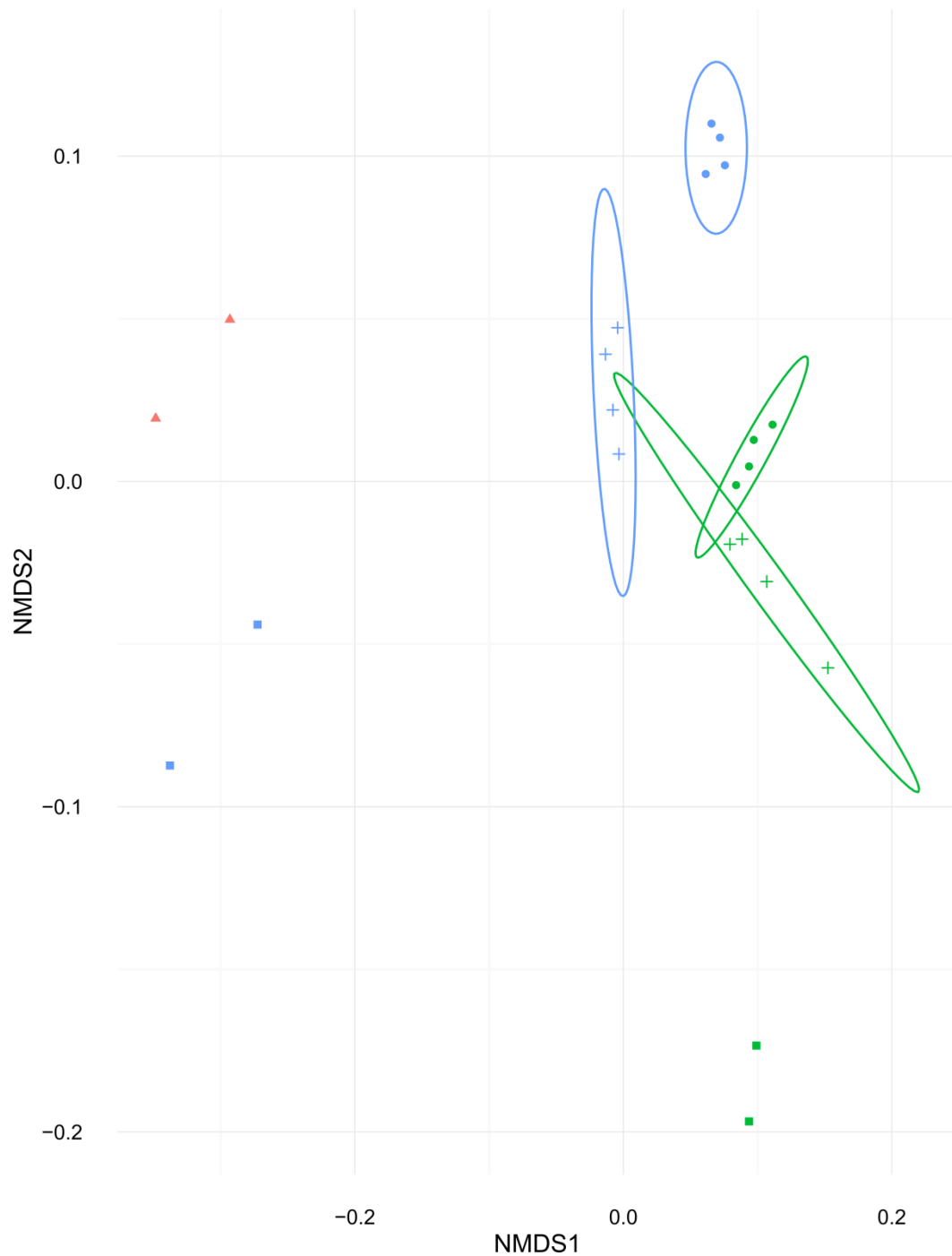
Populations of *Archaea* significantly increased only on the anode of SS-MFCs (6%) compare to SS inoculum (2%) and SW (0.5%) (Fig 2C-D). The most abundant genera of *Archaea* were *Methanosarcina* on anode of SS-MFCs and *Methanothermobacter* on anode of IGBS-MFCs (Fig 2C-D).

## 260 MFC planktonic microbial communities

261 Analysis of the SS-MFC planktonic community showed that phyla *Bacteroidetes* (30%),  
262 *Firmicutes* (25%), *Proteobacteria* (22%), *Actinobacteria* (3%) and *Archaea* (7%) were highly  
263 abundant (Fig 2A). The dominant *Gammaproteobacteria* in inoculum and SW shifted to the  
264 *Deltaproteobacteria* in the MFC planktonic microbial communities (Figs 1A-C and 2A). Members of  
265 phylum *Archaea* were enriched in the planktonic population similarly to population of MFC anodic  
266 surface (Fig 2A-B). The planktonic ~~community-communities~~ of the IGBS-MFCs were similar to their  
267 anodic microbial ~~community-communities~~ (Fig 2B-D). Dominant phyla *Proteobacteria* (49%),  
268 *Firmicutes* (12%), *Bacteroidetes* (12%), *Chloroflexi* (4%), *Archaea* (10%) and *Actinobacteria* (2%)  
269 were found in the planktonic community of IGBS- MFCs (Fig 2).

## 270 Similarity- and phylogeny-based MFC microbial community profiling

271 To determine the relationship between MFC anodic and planktonic microbial communities,  
272 swine wastewater and inocula a two-dimensional ordination plot based on taxonomy was created (Fig  
273 3). Statistically significant dissimilarities were observed across the SW and anodic and planktonic  
274 communities of both MFCs.



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**Figure 3. Ordination plots of a non-metric multidimensional scaling (NMDS) for the microbial communities from SW (inflow), SS- and IGBS-inoculated MFCs.** Blue color indicates microbial communities derived from SS, green color indicates microbial communities derived from IGBS, red color indicates microbial communities derived from SW (circles, anodic microbial communities; crosses, planktonic microbial communities; squares, microbial communities of inoculums; triangles, SW microbial community). NMDS was based on Bray-Curtis distances of prokaryotic species abundance.



283

284 Each sample type can be seen to form a distinct cluster with the IGBS-MFC anodic and  
285 planktonic communities overlapping and the SS-MFC anodic and planktonic communities in close  
286 proximity. The SS and IGBS inoculum communities are well separated from each other. Thus, anodic  
287 and planktonic communities of IGBS-MFCs and SS-MFCs clustered close to one another, while SW  
288 samples did not (Fig 3).

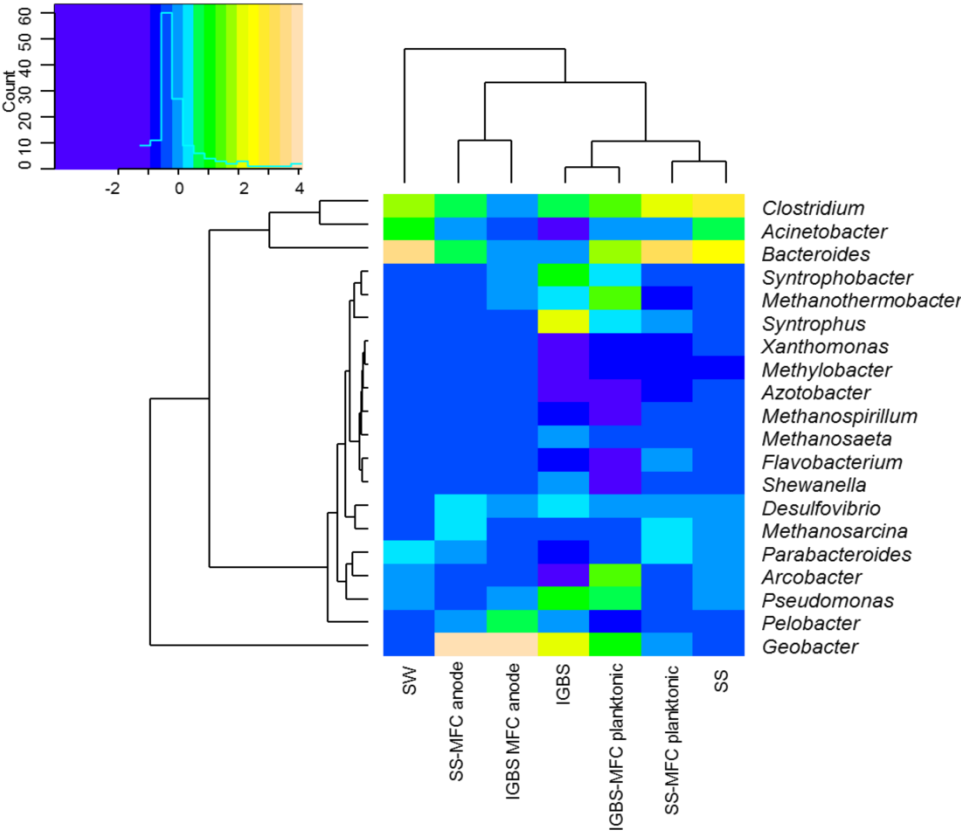
289 A heat map of dominant bacterial genera based on a hierarchical clustering analysis was created  
290 to confirm the similarity and differences between the MFC anodic and planktonic microbial  
291 communities, swine wastewater and inocula (Fig 3). The planktonic MFC communities have a high  
292 similarity with their inoculum communities. These planktonic-inoculum clusters form a secondary  
293 cluster with each other. The MFC anodic communities form their own distinct cluster which contains  
294 *Geobacter* spp., a well-known genus of electrogenic bacteria. The SW community differed from all  
295 microbial communities and formed a separate cluster (Fig 3). Clustering of microbial communities  
296 based on dominant bacterial genera indicates that the electrogenic communities in the MFC developed  
297 from their inocula.

## 298 **Diversity of dominant microbial species in MFC anodic microbial communities**

299 Detailed analyses revealed five abundant genera of *Proteobacteria* enriched on the anodes of  
300 MFCs. The genus *Geobacter* was represented by eight predominant and three minor species in both  
301 MFC anodic communities (Fig 4). Highly abundant *Pelobacter* genus (*Pelobacter propionicus*,  
302 *Pelobacter carbinolicus*) were identified in both anodic communities of MFCs (Fig 4). The diversity  
303 observed within the genus *Desulfovibrio* was significant (Fig 4). *Gammaproteobacteria* were  
304 represented by six dominant bacterial genera in the anodic communities of the SS-MFCs and IGBS-  
305 MFCs. The *Acinetobacter* genus was represented by four abundant species in anodic microbial  
306 communities of both MFCs. Diversity of *Pseudomonas* members associated with MFCs anodes was  
307 tremendous. Among them *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* were the most



308 abundant species in the anodic biofilms of the IGBS-MFCs and SS-MFCs, respectively. The  
 309 *Azotobacter* genus was dominated by *Azotobacter vinelandii* on anodes of both MFCs. Six abundant  
 310 species of *Xanthomonas* genus (dominant *Xanthomonas campestris*) were identified in the anodic  
 311 biofilms of both MFCs. Twenty different members of *Shewanella* genus were found in anodic biofilms  
 312 of both MFCs and *Shewanella baltica* was the most abundant specie among them. One member of  
 313 *Methylobacter* genus (*Methylobacter tundripaludum*) was enriched on anodes of both MFCs (Fig 4).



314  
 315 **Figure 4. Heatmap diagram visualize the dominant bacterial and archaeal genera in the**  
 316 **microbial community profiles.** Bottom represents the different samples.

317 Among *Firmicutes*, over 50 species of the genus *Clostridium* were identified from the MFC  
 318 anodes, including 31 abundant species (dominant *Clostridium thermocellum*) and 9 relatively abundant  
 319 species. *Flavobacterium johnsoniae* and *Flavobacterium psychrophilum*, *Bacteroides fragilis* and  
 320 *Parabacteroides distasonis* were the three dominant bacterial species among *Bacteroidetes* in the  
 321 anodic communities of both MFCs. Acetoclastic methanogens (*Methanosarcina barkeri* and

322 *Methanothermobacter thermautotrophicus*) belonging to the domain of *Archaea* were identified in  
323 MFC anodic and planktonic populations (Fig 4).

## 324 **Discussion**

325 This study demonstrated the compositions and phylogenetic distributions of SW, inocula, anodic  
326 and planktonic microbial communities in SS- and IGBS-inoculated MFCs. The results showed  
327 insignificant differences in bacterial richness and diversity between microbial communities both  
328 MFCs, while SW differed significantly.

### 329 **MFCs treatment efficiency of SW**

330 Treatment of SW using MFCs inoculated with two different inoculums achieved substantial  
331 COD removal rates. A previous study found that a single-chambered MFC with a working volume 28  
332 mL removed only 27% of the COD in SW having a high initial COD of 8,320 mg L<sup>-1</sup> after 44 h (Min  
333 et al., 2005) whereas we found 76.4% and 65.7% removal of COD from SW by the SS-MFCs and  
334 IGBS-MFCs, respectively, after 48 h (data not shown). The average current density of the MFCs (Fig  
335 1, Table 1) were within the range reported for other wastewater-fed MFC systems [5]. Consistent with  
336 other reports [13, 14], differences in external resistance within the range we tested (10 -1000  $\Omega$ ) did  
337 not notably alter the performance of the MFCs (data not shown).

338 Swine wastewater is characterized by high content of VFAs although their initial concentration  
339 in raw swine wastewater across different farms can vary substantially. Our results are consistent with  
340 others demonstrating that MFC treatment of SW largely eliminates VFAs, which are largely  
341 responsible for the SW odor [4]. Importantly, the SW feed in our experiments was approximately 5-  
342 fold higher strength and the HRT less than five times that utilized by Kim et al. [4] and yet the MFCs  
343 still performed well at removing the VFAs.

344 In summary, use of SS as an anodic inoculum resulted in superior treatment performance of the  
345 MFCs over the 67 d course of the experiment compared to IGBS inoculum. This may indicate a more  
346 general tendency of pre-adapted inocula to perform better at degrading the substrate [15].

## 347 **The microbiome of electrogenic of anodic biofilms and planktonic populations of** 348 **MFCs**

349 We used metagenomic analysis to explore the whole taxonomic diversity of the SS- and IGBS  
350 inoculums, SW, anodic and planktonic microbial communities of MFCs. *Proteobacteria* (mostly  
351 *Deltaproteobacteria*) became the predominant phylum in both MFCs anodic communities, while  
352 *Firmicutes* and *Bacteroidetes* decreased. The planktonic community of the IGBS-MFCs showed  
353 notable variation in relative abundance and became more similar to their anodic communities. In  
354 contrast, the planktonic communities of the SS-MFCs were intermediate between the SW and anodic  
355 communities. A previous study of a distillery wastewater-treating pilot-scale MFC inoculated with  
356 IGBS showed that the dominant anodic phyla (*Proteobacteria*, *Bacteroidetes* and *Firmicutes*) were  
357 similar to that of the IGBS inoculum [16].

358 Previous studies have shown that SW could be used as a suitable inoculum for electricity  
359 production using MFCs, distinguished by the chamber and cathode types [4, 17]. Analysis of the  
360 anodic microbial communities in the SS-MFCs mainly showed that dominant species belonged to three  
361 major phyla *Proteobacteria*, *Bacteroidetes* and *Firmicutes* [9, 12]. Results of metagenomics analysis  
362 in our study are in good agreement with results in the literature [9, 12].

363 Detailed analysis of the dominant anodic bacterial species in SW-treating MFCs showed high  
364 diversity in members of the *Deltaproteobacteria*, *Gammaproteobacteria*, *Firmicutes*, *Bacteroides* and  
365 *Archaea*. Among all *Deltaproteobacteria*, *Geobacter metallireducens*, *Pelobacter propionicus*,  
366 *Desulfovibrio vulgaris*, *Syntrophobacter fumaroxidans* and *Syntrophus aciditrophicus* were found to  
367 be the most abundant in the anodic microbial communities of both MFCs. The well-known  
368 electrogenic *Geobacter sulfurreducens*, dominant in the MFC microbial biofilms, generates a current  
369 via membrane c-type cytochromes (*omcZ*) and secretion of pili encoded by the *pilA* gene [3, 18, 19]. In

370 contrast, anoditrophic Fe(III)-reducing *Pelobacter carbinolicus* was characterized as a non-  
371 electrogenic symbiotic bacterium responsible only for converting of substrates to acetate and hydrogen  
372 for use by *G. sulfurreducens* [3, 20]. Cytochrome *c* localized on the outer cell membrane of  
373 *Desulfovibrio desulfuricans* contributed to the electron transfer in an electricity-generating MFC [21].

374 Members of *Deltaproteobacteria* might contribute to VFA degradation. Our data demonstrate a  
375 relative abundance of *Syntrophobacter fumaroxidans* and *Syntrophus aciditrophicus* on the MFC  
376 anodes, which may aid in metabolism of propionic and butyric acid in the SW. Pure culture  
377 experiments with *Geobacter* species isolated from swine wastes were examined the ability to  
378 biodegrade individual and mixtures of VFAs [22]. It was shown that *G. metallireducens*, *G.*  
379 *humireducens* and *G. grbiciae* consume VFAs and stimulate VFAs oxidation depending on availability  
380 of Fe (III).

381 This study demonstrates that *Acinetobacter baumannii* and *Pseudomonas fluorescens* belonging  
382 to *Gammaproteobacteria* were prevalent members in the anodic community both MFCs. The  
383 *Gammaproteobacteria* possess diverse metabolic capabilities involved in a breakdown of different  
384 substrates and production of soluble redox active compounds, resulting in current generation in MFCs  
385 [3, 23, 24]. *Acinetobacter* species dominating in the microbial community of MFCs fed with  
386 fermentable substrates were able to produce electricity [25]. Production of *pili*-like structures encoded  
387 by *csuC* and *csuE* genes in *A. baumannii* influences the colonization of different abiotic surfaces [26].  
388 We found a considerable number of *Pseudomonas* species in both MFCs types. The ability of  
389 *Pseudomonas* to consume various carbon sources is known. Moreover excretion of soluble  
390 electrochemically redox mediators participating in the electricity production in MFCs has been  
391 observed [23, 27]. Thus, dominant *Pseudomonas fluorescens* might be responsible for COD removal  
392 from the SW and the excretion of redox mediators contribute to the observed electricity generation of  
393 the MFCs.

394 The relatively high abundance of *Shewanella baltica* in the anodic microbial communities  
395 provides evidence of their importance in the conversion of COD into electricity. Previous studies have

396 showed that electrogenic *Shewanella* species might transfer electrons to the anodes of MFCs either  
397 through nanowires or excretion of redox active second metabolites [28, 29].

398 Our study demonstrates a relative abundance of bacteria related phyla *Firmicutes* and  
399 *Bacteroidetes*. It is well known that *Clostridium* species participate in fermentation processes and  
400 conversion of organic substrates to VFAs and hydrogen and that they are indigenous microbiota of the  
401 swine gastrointestinal tract and manure [12]. *Bacteroidetes* are widely recognized as the intestinal  
402 microflora associated with fermentation of carbohydrates and utilization of nitrogenous compounds, as  
403 well odor production [30]. We found that the remaining dominant bacteria, *Flavobacterium johnsoniae*  
404 and *Bacteroides fragilis* became even more abundant in the planktonic populations of both MFCs.

405 16S rRNA sequence analysis of a SW-treating MFC microbial community showed that two  
406 members of *Firmicutes*, a gram-positive *Turicibacter* sp. and *Sedimentibacter* spp., were the dominant  
407 genera on the anodes of a MFC having a maximum power point tracking system [9]. Earlier studies  
408 demonstrated reduction of VFAs level depending on a seasonal shift of *Bacteroidetes* members in an  
409 anaerobic lagoon used for swine waste treatment [12].

410 In our study, two archaea species *M. barkeri* and *M. thermautotrophicus* increased in the  
411 bacterial communities of SS-MFCs and IGBS-MFCs, respectively. Rotaru et al. established that the  
412 acetoclastic methanogen *M. barkeri* in association with electrogenic bacterium *G. metallireducens*  
413 participates in direct interspecies electron transfer (DIET) [31]. We found a potential for a DIET-type  
414 bacterial association between *M. barkeri* and *G. metallireducens* in the anodic microbial community of  
415 the SS-MFCs; possible association between *M. thermautotrophicus* and *G. metallireducens* was found  
416 in the anodic microbial community of IGBS-MFCs.

417 Taken together, the profiling of microbial community diversity based on similarity and  
418 phylogeny supports a model for development of electrogenic biofilm in MFCs from their inocula.

419

420

## Conclusion

This research demonstrates the importance inoculum source on the electrogenic and degradative activities and ultimate microbial community composition of SW-treating MFCs. MFC treatment of SW is a potentially more environmentally friendly alternative to energetically costly aerobic treatment or odorous space-demanding anaerobic lagoons. Our comprehensive analysis of SS- and IGBS-inoculated MFCs treating SW revealed stable electricity production by both types of MFCs with the SS-MFC showing the highest current density. Both MFC pairs displayed moderate removal of ammonia, did not remove phosphate, and were highly efficient at removing COD and odiferous VFAs. Analysis of microbial communities of both MFCs showed that MFC anodic communities form their own distinct cluster which contains *Geobacter* spp., represented by eight predominant and three minor species, in both MFC anodic communities. Clustering of microbial communities based on dominant bacterial genera indicates that the electrogenic communities in the MFCs developed from their inocula. The MFC environment enriched for a spectrum of bacterial genera, including *Pelobacter*, *Pseudomonas*, *Arcobacter*, *Syntrophus*, *Syntrophobacter*, *Bacteroides*, *Clostridium* as well as two acetoclastic methanogens (*Methanosarcina* and *Methanothermobacter*).

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## Conflict of Interest

445 The authors declare no conflict of interest.

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## 543 **Supporting Information**

544 **Fig. S1. Cell voltage and power density vs. current density (cell polarization) of MFCs (A)**  
545 **inoculated with swine wastes; (B) inoculated with brewery sludge.** Open circles, voltage; closed  
546 circles, power density.

547 **Fig. S2. Current generation during swine wastewater treatment by MFCs inoculated with swine**  
548 **waste sludge and brewery sludge.** Mean data from duplicate experiments; error bars indicating  $\pm$ SD  
549 to not exceed the diameter of the data point symbols. Open boxes, SS-inoculated MFC; open  
550 diamonds, IGBS-inoculated MFC.

551 **Fig. S3. Total COD concentrations in SW feed and within MFCs inoculated with swine waste**  
552 **sludge and industrial granular brewery sludge.** Squares, SS-inoculated MFC; circles, IGBS-  
553 inoculated MFC; Mean data from duplicate experiments.

554 **Fig. S4. Change in total phosphorus ( $\text{PO}_4^{3-}$  -P) in inflow and outflows of MFCs inoculated with**  
555 **swine waste sludge and industrial granular brewery sludge.** Mean data from duplicate experiments  
556 with error bars ( $\pm$ SD).

557 **Fig. S5. Changes in ammonia ( $\text{NH}_4$ -N) in inflow and outflows of MFCs inoculated with swine**  
558 **wastes sludge and brewery industrial granular sludge.** Mean data from duplicate experiments with  
559 error bars ( $\pm$ SD).

560 **Fig. S6. SEM images of the anodic biofilms the MFCs inoculated with (A) swine waste and (B)**  
561 **brewery sludge.** Samples of anode surfaces (activated carbon granules and fiber) the MFCs were  
562 taken after 67 days of swine wastewater treatment upon disassembling the MFCs. Slices of anode  
563 electrodes ( $1\text{ cm}^2$ ) were briefly rinsed with deionized water and fixed in 2.5% glutaraldehyde for 2 h,  
564 further in 1% osmium tetroxide. Dehydration of microbial biofilms was carried out using a series of  
565 ethanol–water solutions (25, 50, 75, 95, 100%). After gold coating, the obtained specimens were  
566 observed using a Focused Ion Beam Scanning electron microscope (Helios NanoLab 650, USA). High

567 resolution images were acquired using an accelerating voltage of 20 kV at a working distance of 3.1–  
568 6.5 mm.

569 **Table S1. Summary of MFC operation modes.**

570 **Table S2. Concentration of VFAs in the SS-inoculated MFC.**

571 **Table S3. Concentration of VFAs in the IGBS-inoculated MFC.**

572 **Table S4. Diversity of dominated species in the SW, inocula, anodic and planktonic microbial**  
573 **communities of SS- and IGBS-inoculated MFCs.**